Germination Growth Test: Anoxic Conditions

Draft Standard Operating Procedure for Test Methods using Wild Rice, *Zizania palustris*

1.1 Scope and Application

1.1.1 This method describes the procedures developed to perform a toxicity test to determine the response of conditioned *Zizania palustris* (Wild Rice) seedlings to various concentration of dissolved sulfide in an anoxic growth medium.

1.1.2 This method consists of a toxicity test using a dilution series of at least four dissolved sulfide concentrations in a growth medium plus a negative control where no sulfide is present in the medium.

1.2 Summary of Method

1.2.1 Seeds of the aquatic macrophyte *Z. palustris* are conditioned for germination and exposed in a static- renewal system to a dilution series of four concentrations of sulfide. The exposure duration of the seeds is 10 days. The response of the germinating seeds is measured in terms of differences in germination rate and certain other growth parameters between treatments groups and between treatment and control groups.

1.3 Quality Control Considerations

1.3.1 Substances toxic to the seedlings may be introduced by contaminants in dilution water, sampling hardware, or testing equipment.

1.3.2 Effects of pH changes and cationic constituent concentrations in test media may augment or mask effects of toxic substances.

1.3.3 Improper preparation and sampling of test solutions may adversely affect test results (see section *1.5: Standards and Reagents* and section *1.6:Toxicity Test Procedures*)

1.3.4 Additional quality control and quality assurance details for this method can be found in the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfide- Quality Assurance Project Plan" as its implementation relates to the Minnesota Pollution Control Agency's Wild Rice Sulfate Standard Study.

1.4 Necessary Apparatus and Materials

1.4.1 Seeds of *Z. palustris* are prepared in the laboratory for germination (see section *1.7: Wild Rice Seed Preparation*). To initiate exposures, sufficient numbers of conditioned seeds must be available. Each test container (jar) contains 50 conditioned seeds.

1.4.2 Environmental Growth Chamber: Temperature control range of 15° C to 30° C ± 1°C). Germination growth tests are performed in the dark.

1.4.3 Test chambers: 700 mL borosilicate glass jars capped using phenolic screw caps with chlorobutyl septa (Wheaton).

1.4.4 Meter: pH for routine physical measurements.

1.4.5 Volumetric flasks and graduated cylinders: class A, 10 – 2000 mL borosilicate glass for preparation of test solutions.

1.4.6 Volumetric pipets

1.4.7 Glass pipets

1.4.8 Pipet bulbs and fillers

1.4.9 Balance: analytical, capable of accurately weighing 0.1 mg.

1.4.10 Magnetic stirrer and stir bars: for mixing test and growth media solutions.

1.4.11 Filtering apparatus: for membrane and /or glass fiber filters.

1.4.12 Tape: for labeling test chambers and containers for solutions.

1.4.13 Water purification system: deionized water or equivalent.

1.5 Standards and Reagents

1.5.1 Reagent-grade chemicals are used to prepare hydroponic growth media, which consists of a modified Hoagland's nutrient solution,

1.5.2 A modified 1/5 concentration Hoagland's stock nutrient solution, as determined beneficial for wild rice growth, is prepared as part of the test media by using a ½ concentration stock Hoagland's solution daily or more often as needed from a 1.0 M stock solution. (see Table 1 for 1/5 Hoagland's stock solution nutrient composition and concentrations)

1.5.3 PIPES buffer is added to the test media to maintain consistent pH levels throughout an experiment.

1.5.4 Stock sulfide solutions (20 – 30 mM) are prepared as needed by adding $Na_2S \cdot 9\ H_2O$ (Sodium Sulfide Hydrate) to deionized and degassed water.

1.5.5 The hydroponic media is degassed and made anoxic in preparation for use in tests by bubbling nitrogen gas through the solution for at least 24 h. "High Purity" grade nitrogen gas with an attached oxygen scrubber is used for degasification of test the media.

1.5.6 Reagent water: defined as deionized water that does not contain substances that are toxic to the test organisms.

1.5.7 Appropriate amounts of each test solution (700 mL/treatment replicate jar) multiplied by the number of replicates, plus an additional amount required for a sample to be used to characterize the chemistry of the stock test media (approx.1650 mL per treatment for 3 replicates and ~200 mL sample for chemistry characterization) are made up immediately before starting a test. Pre-determined amounts of 1/5 strength Hoagland's, PIPES buffer (Piperazine-N,N'-bis(2-ethanesulfonic acid) sesquisodium salt, Fisher Scientific/Acros Organics # AC32778-5000). The pH is adjusted to 6.8 +/- 0.2 with 1 M HCl.

Table 1. Composition of test media (Modified 1/5 Hoagland's Solution + PIPES Buffer +EDTA)

	Compound	Molar Concentration of 1/5th strength Hoagland's Growth Solution
Hoagland's Solution	$MgCl_2$	0.4 mM
	$CaCl_2 \cdot 2\ H_2O$	2 .0 mM
	KCl	1.0 mM
	H_3BO_3	22.5 µM
	$MnCl_2 \cdot 4\ H_2O$	4.5 µM
	$ZnSO_4 \cdot 7\ H_2O$	0.5 µM
	$CuSO_4 \cdot 5\ H_2O$	0.15 µM
	MoO_3	0.07 µM
	Fe-EDTA	45.0 µM
	$Na_2SiO_3*9H_2O$	1.5 mM
	PIPES buffer	5.0 mM

1.6 Toxicity Test Procedures: Toxicant Exposures

1.6.1 Each toxicity test will consist of at least four toxicologically relevant test concentrations of sulfide in the hydroponics media (hydroponics medium + sulfide) and a negative control which consists of the hydroponics medium without any sulfide added.

1.6.2 Each of the four exposure concentration levels and control are replicated three times, using the same 700 ml glass jars for each set of replicates.

1.6.3 Conditioned wild rice seeds as described in section 1.7.:*Wild Rice Seed Preparation*, are the test organisms used to initiate the toxicity test.

1.6.4 Conditioned seeds (50) are removed from the pool of initial seeds (section 1.8, *Test Organisms*) using a light forceps and put into the jar corresponding to the first integer read from the random integer table. This is done for all jars prepared for testing.

1.6.5 A total of 50 seeds are selected and removed individually from a pool of conditioned seeds (section 1.8, *Test Organisms*) using a light forceps and are placed into a uniquely numbered 700 ml glass jar not yet associated with a treatment level or replicate. This seed placement procedure is conducted using a randomization scheme, where a seed is placed into a jar corresponding to the first integer read from a random integer table. This randomization is done for all jars prepared for testing.

1.6.6 Each jar is labeled with tape using a unique descriptor for describing the particular concentration level of sulfide in the test solution and replicate for that jar.

1.6.7 The seeds and jars are place in a nitrogen tent for about two hours to assure anoxic conditions. Each labeled jar is filled to the top with the prepared (anoxic) hydroponic media solution and capped. The jars are then recapped.

1.6.8 Exposure jars are spiked with a volume of concentrated sulfide solution appropriate for the corresponding nominal sulfide concentration of that jar. Control exposure jars are spiked with degassed distilled water. Sulfide solutions are spiked into the jars through the chlorobutyl septa in the cap using Hamilton volumetric glass syringes. Spike volumes range between 0.2 – 3 mL of spike solution depending on target exposure concentrations and nominal concentration of stock sulfide solution concentration.

1.6.9 The jars are inverted twice to mix the solutions and are placed onto a tray.

1.6.10 Screw caps are placed tightly on the jars.

1.6.11 The experimental jars, now each containing the conditioned seeds, hydroponic test media, and sulfide exposure, are placed onto a tray and stored in a growth chamber without light for the duration of the 10 day experiment, with the exception of being taken out for the renewal of test media.

1.6.12 Test solutions in the jars are renewed every two (2) days.

1.6.13 During a test media renewal day, the caps of the jars are first removed to allow collection of a sample for the immediate measurement of pH in the old solution. Additional samples are then taken of the old solution and preserved for analytical chemistry analysis of sulfide concentration. Proper sampling techniques, preservation methods, and sample labeling procedures are followed as required by the laboratory contracted for analysis. Any remaining old test media not used for pH or analytical chemistry is gently poured off, leaving approximately one vertical cm of old media in the bottom of the jar.

1.6.14 The addition of new hydroponic test media to jars is completed by first degassing the empty jar using a flow of nitrogen sufficient to replace three jar volumes (about 2100 mL). This is completed for each jar as is it emptied. Newly prepared test media is added by siphoning from the container of stock solution until it reaches the top of the jar. This occurs as the jar is continuing to be degassed. The screw cap is then replaced on the jar.

1.6.15 Chemistry as described in the section *1.12: Analytical Chemistry*.

1.6.16 Duration of the experiment is 10 days with test media renewals occurring on 4 of the 10 days.

1.7 Wild Rice Seed Preparation

Wild rice seed must undergo a conditioning phase following its harvest from the field. In the wild, wild rice drops into the water after the seed has ripened, and sinks to the sediment. This seed, if left undisturbed, stays on or just below the surface of the sediment over the winter. This cold phase serves to condition the seed to enable it to germinate once water temperatures increase in the spring.

1.7.1 The following is a procedure that describes the method and handling of wild rice seed from initial harvest to its use in the germination growth toxicity tests.

1.7.2 Freshly harvested seed should be kept cool and moist and be placed into storage as soon as possible after field collection.

1.7.3 Harvested seed prepared for storage can be kept a) in air tight bags in a cooler set at just above freezing (4° C), or b) submerged in water just above freezing in the dark. Seed stored in either manner can have satisfactory germination rates for one to two years.

1.7.4 To begin the seed conditioning for germination, an aliquot of seed (approximately 2000 seeds) is removed from this 'dry' cold storage (as described in option (a) in 1.7.3) and placed into a container with water and kept submerged at near freezing

temperatures for at least one month. Following this time period, seed is ready (or conditioned) for germination for at least several months. For purposes of use in laboratory testing, seed set in this conditioning phase are kept for up to two months before a fresh aliquot of seed is brought into the conditioning phase. Use of storage option (b) keeps the seed in this wet, cold conditioned phase until needed for testing.

1.8 Test Organisms, Conditioned Wild Rice Seed

1.8.1 Conditioned seeds are selected from the pool of available seeds and placed into a separate container with water. Seed are selected and used for initiating a toxicity test by visually screening for viability based on the color of the seed coat and fullness of the seed body. Seeds that float, are misshaped, or are otherwise malformed are not used for testing. See Image 1.

1.8.2 A total of 1000 seeds are selected with 900 used for initiating the toxicity tests, and 100 seeds put aside to be dried and weighed to measure initial weight. Procedures for measuring dry weight are described in "Total Plant Biomass (Dry Weight) Methods" found in Appendix C of the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan".

1.8.3 Germination tests are initiated using this conditioned seed. Cold and submerged conditioned seeds are selected and immediately placed onto an open tray next to their randomly chosen test jars. The seed is then placed into the appropriate test jars as described in sections *1.6.3* to *1.6.5*.

1.8.4 The seed will begin to sprout in about 2 to 3 days.

1.9 Light, Photoperiod, Temperature and Humidity Test Conditions

1.9.1 Tests are performed in the dark.

1.9.2 Temperature is maintained at 21° C for 16 hours and 19° C during 8 hours.

1.9.3 Test growth chamber is maintained at 85% humidity.

1.10 Phytotoxic Effects

1.10.1 Observations of seeds are made during test solution renewal every 2 days. All abnormalities should be recorded.

1.10.2 Observations should include the date, time, treatment level, and replicate number.

1.10.3 Number of germinated seeds and length of plant growth (mesocotyl growth) are measured after the 10 days of the experiment at test termination for each surviving

germinated seed. Measures of plant length (mesocotyl growth) are achieved by placing the stem on a flat surface and placing a ruler next to the stem with the zero mark next to the stem-root transition. The length from the stem-root transition to the tip of the leaf is measured and recorded to the nearest millimeter. (See image 2)

1.11 Acceptability of Test Results

1.11.1 At least 90% of germinated seeds in control jars must be living based on visual observation at test termination.

1.11.2 Mesocotyl length of germinated seeds from control exposures will be at least 2.0 cm at the end of the 10 day duration of the test.

1.1.3 Control germinated seeds should not indicate any visible phytotoxic or developmental symptoms at any time during the test.

1.12 Analytical Chemistry

1.12.1 Sampling and analysis of solutions used for initiating and renewing test exposures will use the following procedures.

1.12.2 New test solutions – For each exposure concentration an extra jar is prepared as described above for use in sampling initial sulfide concentrations. Initial solution pH is also measured from a sample of this jar.

1.12.3 Old test solutions – When exchanging solution or before decanting the final solution the jar is inverted to mix the solution. A syringe is injected into the jar cap. A slight positive pressure is added into the jar using nitrogen gas pushed through a separate syringe line to allow for approximately 30 mL of solution to flow out. Following this the line is attached to an evacuated serum jar (125 mL) to sample for analytical chemistry.

References

D. R. Hoagland and D.I . Arnon. 1950. The water-culture method for growing plants without soil. Circ. 347. Univ. of Calif. Agric. Exp. Station, Berkley

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U.S. EPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013.

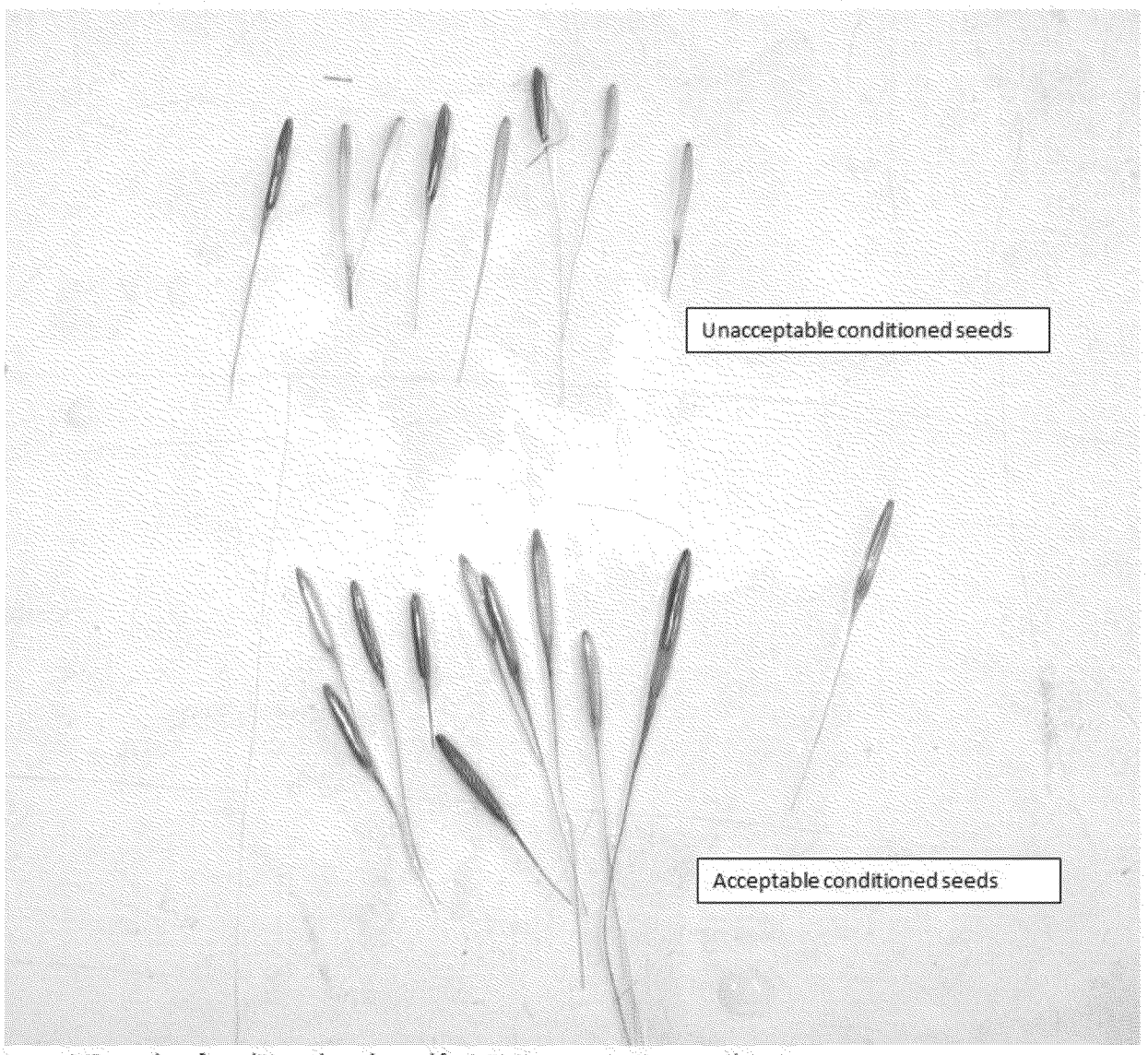

Image 1. Examples of conditioned seeds used for initiating germination growth tests.

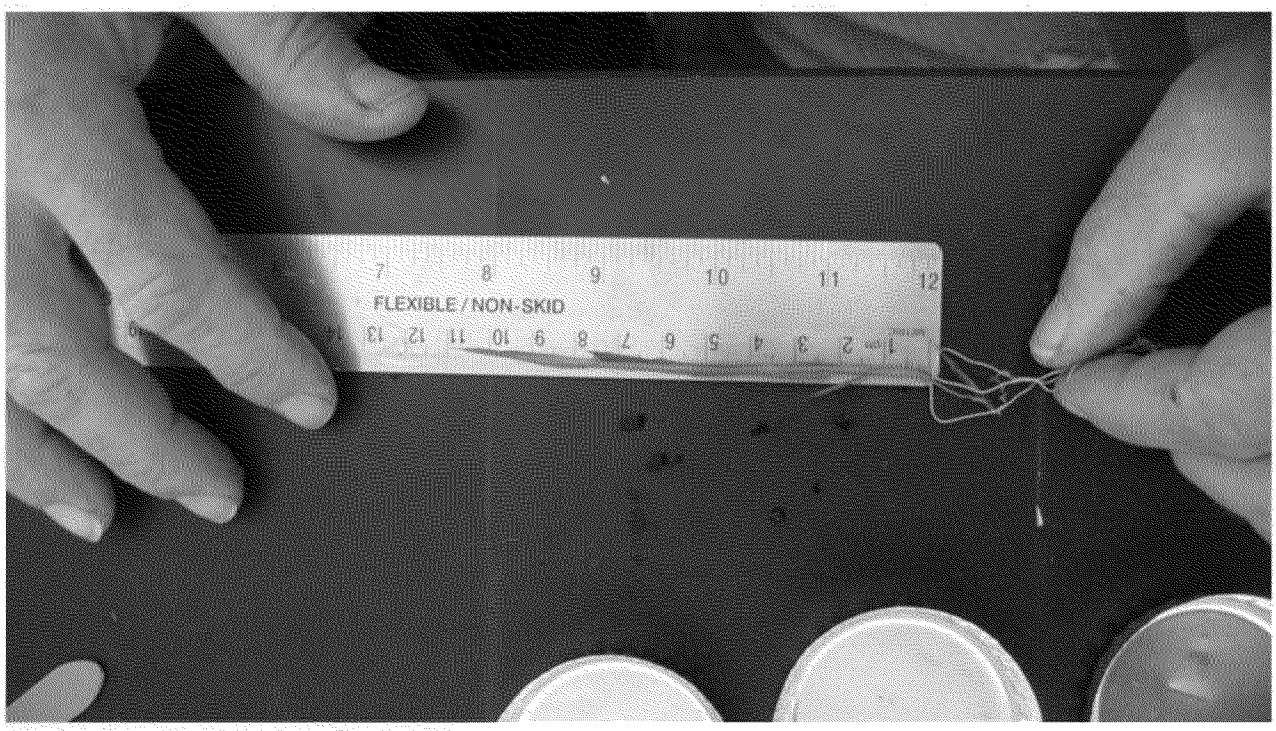

Image 2. Measurement of seedling length.